Heliyon 8 (2022) e08881

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Anxiolytic and antidepressant-like activities of aqueous extract of *Azadirachta indica* A. Juss. flower in the stressed rats

Thaneeya Hawiset [a,*], Napatr Sriraksa [b,c], Utcharaporn Kamsrijai [a], Keerati Wanchai [a], Prachak Inkaew [d,e]

[a] School of Medicine, Mae Fah Luang University, Muang, Chiang Rai, 57100, Thailand
[b] School of Medical Sciences, University of Phayao, Muang, Phayao, 56000, Thailand
[c] Unit of Excellence in the Pulmonary and Cardiovascular Health Care, University of Phayao, Muang, Phayao, 56000, Thailand
[d] School of Science, Mae Fah Luang University, Muang, Chiang Rai, 57100, Thailand
[e] Center of Chemical Innovation for Sustainability (CIS), Mae Fah Luang University, Chiang Rai, 57100, Thailand

ARTICLE INFO

Keywords:
Azadirachta indica A. Juss.
Anxiolytic activity
Antidepressant effect
Restraint stress

ABSTRACT

The aim of this study was to evaluate whether an aqueous extract of *Azadirachta indica* A. Juss. (*A. indica*) flower had anxiolytic and antidepressant-like effects in the stressed rats. Male Wistar rats were randomly allocated to one of two experimental groups: control or stress. The stress groups were received restraint stress for 3 h. The stressed rats were administered a vehicle, diazepam, fluoxetine, and *A. indica* at doses of 250, 500, and 1000 mg/kg BW for 30 days. The elevated plus-maze test (EPMT), the forced swimming test (FST), and the open field test (OFT) were used to assess anxiolytic and antidepressant-like behaviors. In the EPMT, the percentage of the number of open arm entries and the duration spent in open arms were measured. These measurements were considerably enhanced in the stressed rats treated with diazepam and *A. indica* flower extract at a dose of 500 mg/kg BW. Furthermore, the stressed rats given fluoxetine and *A. indica* flower extract at all doses employed in this study showed a significant reduction in the amount of time the rats were immobilized in the FST. However, there was no significant difference in spontaneous locomotor activity between any of the groups. Additionally, the stressed rats treated with either positive control medications or *A. indica* flower extract exhibited significantly higher brain dopamine (DA) and serotonin (5-HT) levels, but lower blood cortisol levels as compared to the stressed rats treated with vehicle. Moreover, *A. indica* flower extract had no harmful effect on the stressed rats' liver tissue.

1. Introduction

Stress is an unpleasant emotion accompanied by changes in biochemistry, physiology, and behavior (Baum, 1990). Chronic stress is linked to structural deterioration and brain malfunction at all stages of life. These abnormalities can lead to an increased risk for neuropsychiatric diseases including anxiety and depression (Mah et al., 2016). Neuropsychiatric disorders are common causes of disability in the world. These disorders affect an estimated 450 million people worldwide (WHO, 2001). Anxiety and depression are the two most common causes of mental disorders among stress-related psychiatric disorders (Whiteford et al., 2013). Nowadays, psychiatric drugs such as monoamine oxidase inhibitors, benzodiazepines, selective serotonin reuptake inhibitors, and tricyclic antidepressants are recommended for relieving symptoms such as excessive tension and guilt. However, the medications in question have negative side effects such as sexual dysfunction, weight gain, and cardiovascular problems (Masand and Gupta, 2002; Rothschild, 2000). As a result, the development of effective anxiolytic and antidepressant treatments based on medicinal herbs with a more beneficial side effect profile is required.

Azadirachta indica A. Juss. (*A. indica*), commonly known as "Neem" or "Sadao" in Thailand, is a member of the Meliaceae family. It is a widely used traditional medicinal plant in Ayurvedic, Chinese, Homeopathic and Unani medicines, particularly in Asia and Africa (Alzohairy, 2016; Eid et al., 2017). In Thailand, young leaves and flowers of *A. indica* are extensively consumed as vegetable and used as a traditional medicine in household remedies for a variety of ailments, including headaches, insomnia, and stress relief. There are various studies on *A. indica* leaf extract in rats. *A. indica* leaf extract has anxiolytic (Jaiswal et al., 1994) and anti-stress (Sen et al., 1992) properties. However, there are limited

* Corresponding author.
E-mail address: thaneeya.haw@mfu.ac.th (T. Hawiset).

https://doi.org/10.1016/j.heliyon.2022.e08881
Received 29 August 2021; Received in revised form 21 October 2021; Accepted 30 January 2022

scientific data on *A. indica* flower extract. In vitro studies have shown that *A. indica* flower extract can scavenge free radicals, and reduce lipid peroxidation in a bronchogenic cancer cell line (Chaisawangwong and Gritsanapan, 2009). In vivo studies, this flower extract could reduce cholesterol solubility in micelles, and modulate gastrointestinal motility (Duangjai et al., 2019). It also helped diabetic rats to lower blood sugar and recover sciatic nerve function (Waliullah et al., 2008; Sriraksa et al., 2019). This extract could protect the kidneys of rats from oxidative stress by enhancing renal anti-oxidative activity (Wanchai et al., 2021). There is scientific evidence to support the effects of *A. indica* flower extract on multiple systems. However, no research of *A. indica* flower extract on the central nervous system has been done. To better understand the influence of *A. indica* on neuropharmacological activities, this study evaluated the effect of *A. indica* flower extract on anxiolytic and antidepressant-like activities in the stressed rats.

2. Materials and methods

2.1. Plant material and preparation of the aqueous extract

The fresh flower of *A. indica* was obtained from Maetumboonyong district, Phayao province, Thailand. The voucher specimen (no. 003805) was deposited in the herbarium of the Faculty of Biology, Naresuan University, Thailand. The sample was authenticated by Dr. Pranee Nangngam, the Faculty of Biology, Naresuan University, Thailand. The flower was washed three times with distilled water. The washed flower was blended, filtered, and dried at 60 °C for 10 h in a forced-air oven. The dried flower was ground into a coarsely powdered extract and then stored at -20 °C until use.

2.2. Animals

Young adult male Wistar rats (age 8 weeks and weights between 200 and 220 g), were purchased from the Nomura Siam International Co., Ltd. (Bangkok, Thailand). During the experimental period, three animals were housed per cage and maintained under a 12-h light/dark cycle. The food and water were provided in containers and the animals could access whenever they needed. The animals were acclimatized to behavior testing equipment for 3 days before they were tested and were randomly divided into various experimental groups ($n = 6$ per group).

2.3. Ethics approval

The Ethics Committee of the Laboratory Animal Research Center, Mae Fah Luang University approved this project on September 20, 2019 (Approval no. AR 03/62).

2.4. Drugs and administration

The animals were assigned into seven groups; one control and six stress groups. The animals in the stress groups were divided into one vehicle group, two positive control groups, and three experimental groups. Animals in the control group had neither stress nor treatment. In the anxiolytic activity tests, the positive control group was administered with diazepam (an anxiolytic drug, manufactured and distributed by the Government Pharmaceutical Organization, Bangkok, Thailand) at a dose of 2 mg/kg, whereas in the antidepressant activity, the positive control group was administered with 5 mg/kg fluoxetine HCl (an antidepressant drug, manufactured and distributed by the Medicpharma CO., LTD, Samut Sakhon, Thailand). Rats in the three experimental groups were treated with *A. indica* flower extract at doses of 250, 500, and 1000 mg/kg BW. All drugs were administered only once daily via oral gavage between 9.00 and 10.00 AM for 30 consecutive days. 30 minutes after the treatment, the animals were immobilized for 3 h to induce restraint stress. The behavioral tests were conducted on Days 1, 15, and 30 after 30 min restraint stress.

At the end of the experiment, the animals were euthanized with 40 mg/kg BW of thiopental sodium via intraperitoneal injection. Subsequently, the blood samples were collected from the heart with the clot blood tube for further analysis of serum cortisol levels. The brains were obtained to determine the monoamine neurotransmitters levels including dopamine (DA) and serotonin (5-HT). The livers were examined gross pathology using hematoxylin and eosin (H&E) staining.

2.5. Acute toxicity study

The thirty-six animals were allocated into six groups (six animals per group). Group I: The animals were treated orally with distilled water (0.5 mL/300 g BW). Group II-VI: The animals were treated orally for one dose with *A. indica* flower extract (250, 500, 1000, 2000, and 4000 mg/kg BW) between 9.00 and 10.00 AM. The oral gavage administration was carried out after a fasting period of 6 h. The symptoms of toxicity include nosebleed, sedation, hypoactivity, loss of appetite (weight loss), dizziness, dyspnea, convulsion, and hyperventilation. The symtoms of toxicity and mortality of the animals were monitored for 24 h after the administration of the doses.

2.6. The behavioral test of the rats

The behavioral tests including the elevated plus-maze test (EPMT), the forced swimming test (FST), and the open field test (OFT) were conducted on Days 1, 15, and 30. The experimental design and protocol were shown in Figure 1.

2.6.1. Elevated plus-maze test (EPMT)

The anxiolytic activity was determined using the EPMT (Pellow et al., 1985). The test apparatus was composed of two enclosed arms (length 50 cm × width 10 cm × height 40 cm), two open arms (length 50 cm × width 10 cm), and a central platform (10 cm × 10 cm). The maze was elevated approximately 50 cm above the floor. Each rat was placed into the center zone by facing one of the open arms and allowed the rat to freely explore the maze for 5 min while being recorded by a video camera. The number the rat entered the open-arm area and the durations the rat spent in the enclosed and open arms areas were also recorded. The rat was considered as entering the open arm area when it placed its four paws on the open arm area. Increasing of numbers of open-arm entries and the duration spent in the open arms indicated that the rat had less anxiety. The maze was cleaned with 70% ethanol solution after each rat had explored the maze. The percentage of the number of open arm entries ($\%n_{open}$) (Thippeswamy et al., 2011) was calculated by Eq. (1)

$$\%n_{open} = \frac{\textit{The number of open arm entries}}{\textit{The total number of enclosed and open arm entries}} \times 100 \qquad \text{Eq. (1)}$$

The percentage of the duration spent in the open arm entries ($\%t_{open}$) (Thippeswamy et al., 2011) was calculated by Eq. (2)

$$\%t_{open} = \frac{\textit{The duration spent in the open arm entries}}{\textit{The duration spent in enclosed and open arm entries}} \times 100 \qquad \text{Eq. (2)}$$

2.6.2. Forced swimming test (FST)

The forced swimming test was used to assess depressive-like behavior (Porsolt et al., 1977). Rats were forced to swim for 5 min in a glass cylinder (height 50 cm × diameter 22 cm) which contained fresh water 33 cm from the bottom at 25 °C. The forced swimming test was recorded with a video camera for further analysis of immobility time and climbing time made by the rats. The rats were considered as immobilized when they floated in the water without struggling and keeping their heads above the water. The rats were considered to have antidepressant-like activities when their durations of immobility time were lower or their durations of climbing were higher than those of the control group.

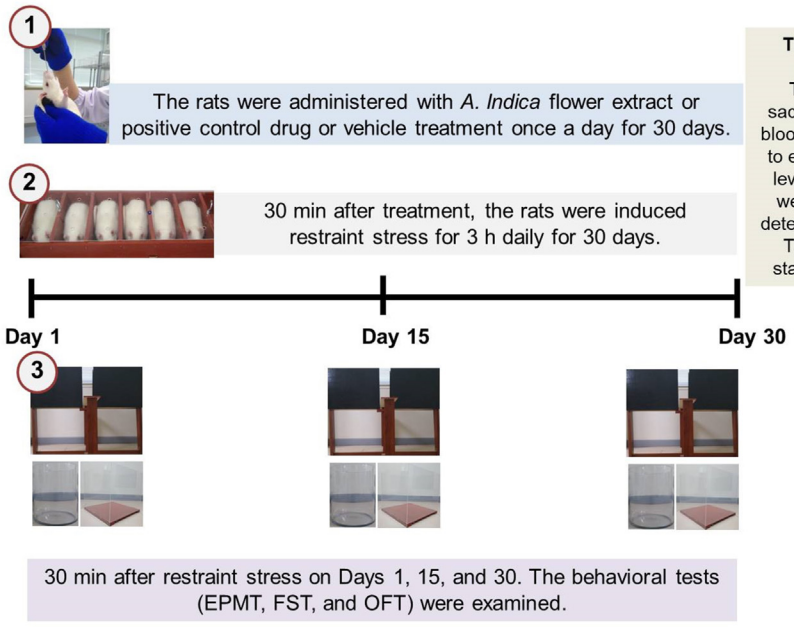

Figure 1. The experimental design and protocol: The rats were orally given either distilled water or positive control medications or *A. indica* flower extract at doses of 250, 500, and 1000 mg/kg BW once a day for 30 days (n = 6 per group). 30 minutes after administration, the rats were induced restraint stress for 3 h daily. The behavioral tests including the elevated plus-maze test (EPMT), the forced swimming test (FST), and the open field test (OFT) were conducted 30 min after restraint stress on Days 1, 15, and 30.

2.6.3. Open field test (OFT)

The open field test is widely used to determine the exploratory behavior and general activity of the rats (Hall, 1934). The OFT was modified and described by Thippeswamy et al. (2011). The apparatus was composed of a clear plexiglass box (length 40 cm × width 40 cm × height 40 cm) with the floor divided equally into 16 squares. Each rat was placed into the center of the field and was allowed to freely explore the area. The number of crossing was evaluated by counting the number the rats moved their positions to stay within a single square with four paws. The number of rearing was evaluated by the number the rats stood on the hind legs. These activities were recorded for 5 min.

2.7. Measurement of blood cortisol levels

At the end of the experiment, blood samples from the heart were collected and centrifuged at 1500 × g for 15 min. The supernatant was collected and stored at -80 °C. The samples were analyzed within 1 month of storing. Cortisol concentrations in serum were diluted in 1:100 ratio. The analysis was performed in duplicates using a cortisol ELISA kit (Catalog No. EK7119, Boster Biological Technology, Pleasanton, USA). The sensitivity limit for the cortisol assay was 17.3 pg/mL. The intra assay and inter assay coefficients of variance were 14.7% and 10.9%, respectively.

2.8. Measurement of brain monoamine neurotransmitter levels

After the behavioral test on Day 30, the rats were sacrificed, and the whole brains were quickly collected and stored at -80 °C for biochemical analysis. The cerebral cortex and the hippocampus were taken from the whole brain. These tissues were weighed and then homogenized in PBS (tissue weight (g): PBS volume (mL) = 1:9) with a glass homogenizer on ice. The homogenates were then centrifuged at 5000 × g for 5 min at 4 °C to get the supernatant. The levels of DA and 5-HT were measured by ELISA (Elabscience Biotechnology Co. Ltd, Catalog No. E-EL-0046, Houston, USA). The intra-assay coefficient of variation of DA and 5-HT were less than 10%. The sensitivities of assays for DA and 5-HT were 18.75 pg/mL and 9.38 ng/mL, respectively.

2.9. Histological measurement

At the end of the experiment, the liver was obtained from each rat. The right lobe of each liver was fixed in 4% paraformaldehyde for 7 days and processed for paraffin embedding. Sections were cut using a microtome and stained with hematoxylin and eosin (H&E) for 10 min at room temperature. A Carl fluorescence microscope (Axio-Scope, Rushmore Precision Co., Ltd.) with ZEN 2.3 (blue edition) program was used to determine the morphology of the liver at a 10X magnification.

2.10. Statistical analysis

The data were expressed as the mean ± standard error of the mean (S.E.M.). The statistical analysis was performed by one-way analysis of variance (ANOVA) followed by LSD post hoc test using SPSS Statistics 22 software and GraphPad Prism 6 software. The differences between groups were considered as statistically significant at probability values lower than 0.05 ($P < 0.05$).

3. Results

3.1. Acute toxicity study

Administrations of different levels of *A. indica* flower extract ranging from 250 to 4000 mg/kg BW were safe for the rat. There were no rat deaths. However, the rats given *A. indica* flower extract at 4000 mg/kg BW showed sedation and hypoactivity as shown in Table 1.

Table 1. Effect of *A. indica* flower extract at doses of 250, 500, 1000, 2000, and 4000 mg/kg BW and distilled water (0.5 mL per 300 g BW) on toxic symptoms and mortality in rats.

Drug	Dose (mg/kg)	Mortality	Symptoms of toxicity
Distilled water	-	0	None
A. indica	250	0	None
A. indica	500	0	None
A. indica	1000	0	None
A. indica	2000	0	None
A. indica	4000	0	Sedation and hypoactivity

Symptoms of toxicity include nosebleed, sedation, hypoactivity, loss of appetite (weight loss), dizziness, dyspnea, convulsion, and hyperventilation.

3.2. Effect of A. indica flower extract on the changes of morphology and histology of the liver

No abnormality was noticed in the gross pathology of the liver in any of the groups. Morphology and histology evaluation revealed normal histological appearance in all groups as shown in Figure 2.

3.3. Effect of A. indica flower extract on anxiolytic activity

The results from the EPMT were presented in Figure 3(a) and (b). The stressed rats treated with vehicle had lower the percentage of open arm entries (Day 1: $F_{(5,30)} = 1.978$, $P < 0.05$; Day 15: $F_{(5,30)} = 2.939$, $P < 0.05$) and the percentage of duration spent in the open arms in EPMT (Day 1: $F_{(5,30)} = 2.764$, $P < 0.05$; Day 15: $F_{(5,30)} = 2.704$, $P < 0.05$) compared to the control rats. On Days 15 and 30, the stressed rats treated with *A. indica* flower extract at 500 mg/kg BW significantly increased the percentage of open arm entries (Day 15: $F_{(5,30)} = 2.939$, $P < 0.01$; Day 30: $F_{(5,30)} = 1.780$, $P < 0.05$) and the percentage of duration spent in the open arms (Day 15: $F_{(5,30)} = 2.704$, $P < 0.05$; Day 30: $F_{(5,30)} = 1.822$, $P < 0.05$) compared to the stressed rats treated with vehicle. However, the stressed rats treated with the extract at 250 and 1000 mg/kg BW were not statistically different. Moreover, the stressed rats administered with diazepam significantly increased both the percentage of the number of open arm entries (Day 1: $F_{(5,30)} = 1.978$, $P < 0.05$; Day 15: $F_{(5,30)} = 2.939$, $P < 0.05$; Day 30: $F_{(5,30)} = 1.780$, $P < 0.05$) and the percentage of duration spent in the open arms in the EPMT (Day 1: $F_{(5,30)} = 2.764$, $P < 0.05$; Day 15: $F_{(5,30)} = 2.704$, $P < 0.05$; Day 30: $F_{(5,30)} = 1.822$, $P < 0.05$).

3.4. Effect of A. indica flower extract on antidepressant activity

The effect of *A. indica* flower extract on the duration of immobility times in the FST was presented in Figure 4(a). Our study demonstrated that the stressed rats treated with vehicle showed no significant difference in the immobility time as compared to the control rats. Compared to the stressed rats administered with vehicle, the stressed rats administered with *A. indica* flower extract at a dose of 250 mg/kg BW had lower duration of immobility time on Day 1 ($F_{(5,30)} = 7.146$, $P < 0.05$), Day 15 ($F_{(5,30)} = 16.119$, $P < 0.001$), and Day 30 ($F_{(5,30)} = 6.185$, $P < 0.05$). The stressed rats treated with *A. indica* flower extract at a dose of 500 mg/kg BW had significantly shortened durations of immobility time on Day 1 ($F_{(5,30)} = 7.146$, $P < 0.01$), Day 15 ($F_{(5,30)} = 16.119$, $P < 0.001$), and Day 30 ($F_{(5,30)} = 6.185$, $P < 0.01$). Moreover, the stressed rats treated with fluoxetine and the highest dose of *A. indica* flower extract (1000 mg/kg BW) had significantly lower durations of immobility time on Day 1 ($F_{(5,30)} = 7.146$, $P < 0.001$), Day 15 ($F_{(5,30)} = 16.119$, $P < 0.001$), and Day 30 ($F_{(5,30)} = 6.185$, $P < 0.05$).

The effect of *A. indica* flower extract on the duration of climbing times in the FST was demonstrated in Figure 4(b). On Day 1, the stressed rats treated with either fluoxetine or *A. indica* flower extract at a dose of 1000 mg/kg BW required higher duration of climbing time ($F_{(5,30)} = 1.499$, $P < 0.05$) compared to the stressed rats administered with vehicle. On Day 15, the stressed rats treated with fluoxetine and *A. indica* flower extract at doses of 250, 500, and 1000 mg/kg BW had longer climbing time ($F_{(5,30)} = 4.956$, $P < 0.01$, $P < 0.01$, $P < 0.05$, and $P < 0.001$, respectively). On Day 30, the stressed rats treated with fluoxetine showed a significantly increased in duration of climbing time ($F_{(5,30)} = 1.768$, $P < 0.05$) as compared to the stressed rats administered with vehicle.

3.5. Effect of A. indica flower extract on spontaneous locomotor activity

In the open field test, the stressed rats treated with diazepam, fluoxetine, and *A. indica* flower extract at doses of 250, 500, and 1000 mg/kg BW exhibited no significant difference in the number of rearing (Day 1: $F_{(6,35)} = 1.216$; Day 15: $F_{(6,35)} = 0.863$; Day 30: $F_{(6,35)} = 0.902$) and

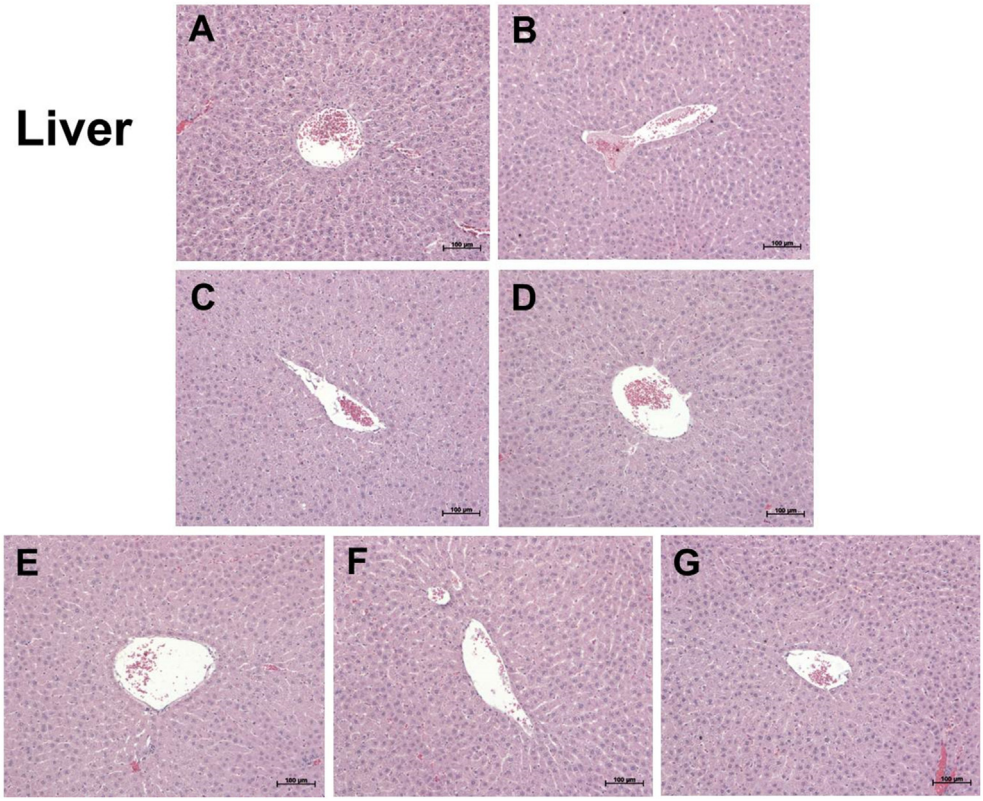

Figure 2. Histopathology of representative sections of liver of rats was determined by hematoxylin and eosin (H&E) at a 10X magnification. A: Control, B: Stress + Vehicle, C: Stress + Diazepam, D: Stress + Fluoxetine, E: Stress + *A. indica* at 250 mg/kg, F: Stress + *A. indica* at 500 mg/kg, G: Stress + *A. indica* at 1000 mg/kg. Scale bar: 100 μm.

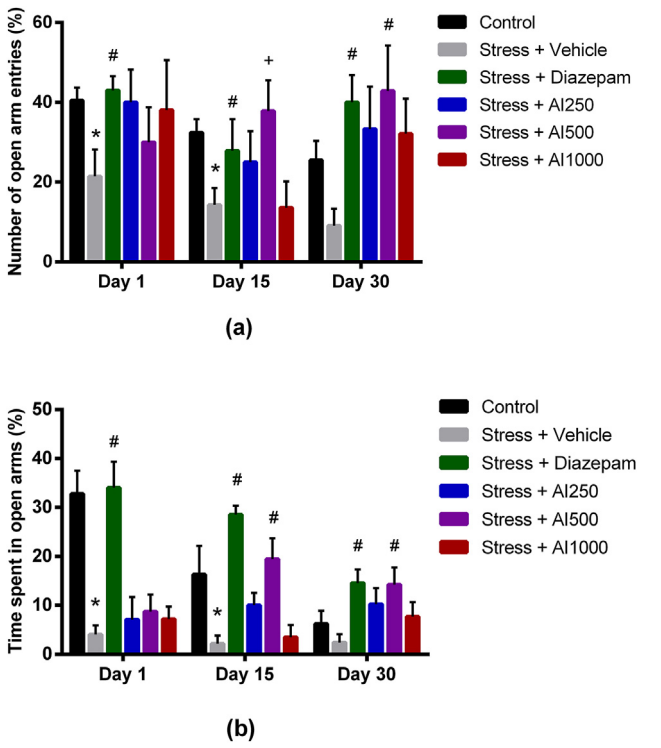

Figure 3. Effects of diazepam and *A. indica* (AI) flower extract (250, 500, and 1000 mg/kg BW) on the anxiolytic-like activity using the EPMT in the stressed rats. (a) The percentage of the number of open arm entries during a 5 min test. (b) The percentage of time spent in open arms during a 5 min test. Data are presented as mean ± S.E.M. ($n = 6$ per group). *$P < 0.05$, as compared to the control group, $^{\#}P < 0.05$ and $^{+}P < 0.01$, as compared to the stressed rats treated with vehicle.

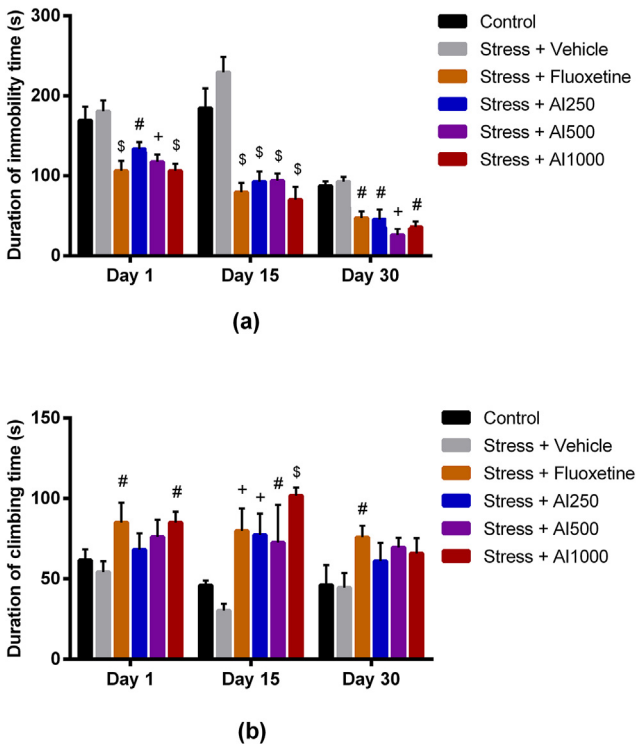

Figure 4. Effects of fluoxetine and *A. indica* (AI) flower extract (250, 500, and 1000 mg/kg BW) on the antidepressant-like activity using the FST in the stressed rats. (a) The duration of immobility time during a 5 min test. (b) The duration of climbing time during a 5 min test. Data are presented as mean ± S.E.M. ($n = 6$ per group). $^{\#}P < 0.05$, $^{+}P < 0.01$, $^{\$}P < 0.001$, as compared to the stressed rats treated with vehicle.

crossing (Day 1: $F_{(6,35)} = 0.461$; Day 15: $F_{(6,35)} = 0.892$; Day 30: $F_{(6,35)} = 0.548$) as compared to the control and the stressed rats treated with vehicle as demonstrated in Figure 5(a) and (b).

3.6. Effect of A. indica flower extract on blood cortisol levels

Blood cortisol levels were presented in Figure 6. Our results reported that the stressed rats administered with vehicle had significantly increased blood cortisol levels ($F_{(6,35)} = 4.262$, $P < 0.05$) when compared to the control group. Moreover, the stressed rats treated with diazepam, fluoxetine, and *A. indica* flower extract at doses of 250, 500, and 1000 mg/kg BW had significantly reduced blood cortisol levels ($F_{(6,35)} = 4.262$, $P < 0.01$, $P < 0.01$, $P < 0.001$, $P < 0.001$, and $P < 0.01$, respectively) when compared to the stressed rats administered with vehicle.

3.7. Effect of A. indica flower extract on the brain monoamine neurotransmitter levels

The results of the brain DA and 5-HT levels measured by ELISA were shown in Figure 7(a) and (b). The stressed rats treated with vehicle had significantly decreased DA levels in both the cerebral cortex ($F_{(6,35)} = 7.317$, $P < 0.05$) and the hippocampus ($F_{(6,35)} = 4.278$, $P < 0.05$) as compared to the control rats. The stressed rats administered with diazepam, fluoxetine, and *A. indica* flower extract at doses of 250, 500, and 1000 mg/kg BW had higher DA levels in both the cerebral cortex ($F_{(6,35)} = 7.317$, $P < 0.001$ in all groups) and the hippocampus ($F_{(6,35)} = 4.278$, $P < 0.01$, $P < 0.001$, $P < 0.01$, $P < 0.01$, and $P < 0.01$, respectively) as compared to the stressed rats treated with vehicle. Figure 7(b) demonstrated that the stressed rats administered vehicle had lower 5-HT levels in the cerebral cortex ($F_{(6,35)} = 4.469$, $P < 0.05$) and the hippocampus

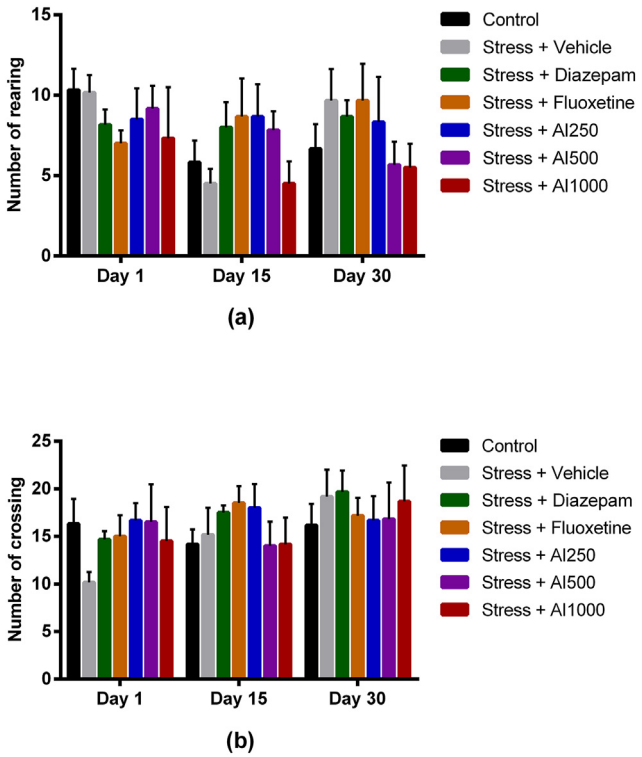

Figure 5. Effects of diazepam, fluoxetine, and *A. indica* (AI) flower extract (250, 500, and 1000 mg/kg BW) on the spontaneous locomotor activity using the OFT in the stressed rats. (a) Number of rearing behavior during a 5 min test. (b) Number of crossing behavior during a 5 min test. Data are presented as mean ± S.E.M. ($n = 6$ per group).

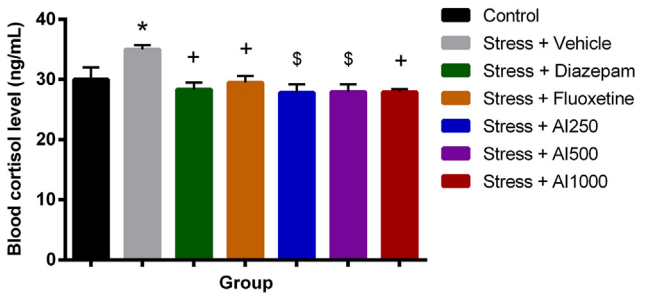

Figure 6. Effects of diazepam, fluoxetine, and *A. indica* (AI) flower extract (250, 500, and 1000 mg/kg BW) on blood cortisol levels. Data are presented as mean ± S.E.M. ($n = 6$ per group). *P < 0.05, as compared to the control group, $^{+}$P < 0.01, $^{\$}$P < 0.001, as compared to the stressed rats treated with vehicle.

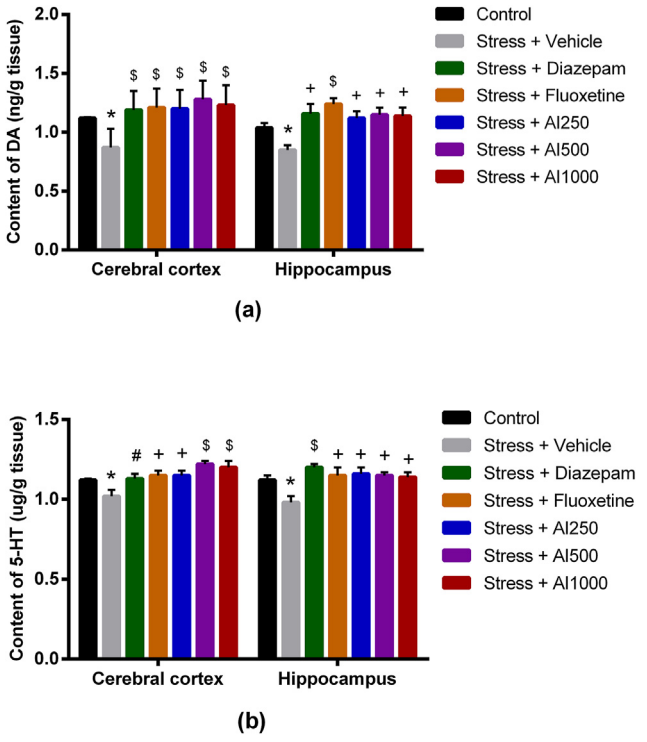

Figure 7. Effects of diazepam, fluoxetine, and *A. indica* (AI) flower extract (250, 500, and 1000 mg/kg BW) on the DA (a) and the 5-HT (b) levels in rat's brains. Data are presented as mean ± S.E.M. ($n = 6$ per group). *P < 0.05, as compared to the control group, $^{\#}$P < 0.05, $^{+}$P < 0.01, $^{\$}$P < 0.001, as compared to the stressed rats treated with vehicle.

($F_{(6,35)} = 3.980$, P < 0.05) as compared to the control rats. Pretreatment of diazepam, fluoxetine, and *A. indica* flower extract at doses of 250, 500, and 1000 mg/kg BW significantly elevated 5-HT levels in the cerebral cortex ($F_{(6,35)} = 4.469$, P < 0.05, P < 0.01, P < 0.01, P < 0.001, and P < 0.001, respectively) and the hippocampus ($F_{(6,35)} = 3.980$, P < 0.001, P < 0.01, P < 0.01, P < 0.01, and P < 0.01, respectively) of the stressed rats when compared to the stressed rats treated with vehicle.

4. Discussion

In the present study, the anxiolytic and antidepressant-like effect of *A. indica* flower extract was investigated in the stressed rats via behavioral, biochemical, and histological approach. Our results demonstrated that *A. indica* flower extract had anxiolytic effect as reported in the EPMT, and an antidepressant effect as determined by the FST. The EPMT is a valid tool to screen anxiolytic drugs for anxiety models in animals, while FST is commonly used to test the efficacy of various antidepressant drugs in rodents (Pellow et al., 1985; Porsolt et al., 1977). The pathogenesis of anxiety and depression are associated with the neurotransmitter dysfunction in the brain (Martin et al., 2009). The monoamine compounds like DA and 5-HT are the main neurotransmitters to regulate emotion and behavior (Martin et al., 2009). The deficiency of these neurotransmitters in the blood and the brain relates to the pathogenesis of anxiety and depression (Martin et al., 2009; Lopez-Munoz and Alamo, 2009; Liu et al., 2018). The large numbers of dopaminergic and serotonergic neuron nerve fibers send the nerve impulses to the cerebral cortex and the hippocampus to release DA and 5-HT to regulate emotion, motivation, and memory (Liu et al., 2018). Hence, the DA and 5-HT levels in the cerebral cortex and the hippocampus can be used to predict pathogenesis of anxiety and depression.

Stress is an extensive life experience that influences the daily well-being of humans. It contributes to various diseases such as anxiety and depression (Mah et al., 2016). Joo and colleagues reported that a 2-h immobilization stress each day for 15 days caused anxiety-like behaviors in mice (Joo et al., 2009). These results are in good agreement with our work which demonstrated that a 3-h daily immobilization stress for 15 days could induce anxiety-like behaviors in rats. However, 3-h immobilization stress could not induce depressive behavior observed via the FST. Suvrathan and co-authors demonstrated that chronic immobilization stress (2 h a day for 10 days) had no significant change in the duration of immobility time, but decreased the climbing time of the stressed rats as compared to the control rats (Suvrathan et al., 2010). The results from our work agreed well with these findings; we found no significant difference in immobility time between the stressed and control rats. Nevertheless, study from Chu and colleagues reported that 24-h restraint stress for 35 days induced long-term depressive-like behavior in mice (Chu et al., 2016). Therefore, the duration and intensity of immobilization stress are important in the development of depressive behavior in rats. This information suggests that evaluating of depression induced by stress should vary intensity and duration of stress for accurate assessment of such factors.

Restraint stress can alter the brain monoamine levels (Matuszewich et al., 2002; Perveen et al., 2003; Sunanda et al., 2000; Torres et al., 2002). Previous study demonstrated that the rats restraint for 1 h significantly increased the levels of DA and 5-HT in the prefrontal cortex and the hippocampus (Matuszewich et al., 2002). Conversely, the rats restraint for 2 h exhibited a significant decrease in the brain DA and 5-HT levels (Perveen et al., 2003). However, if such rats were restraint for 6 h for 3 weeks, they had significantly lower levels of the hippocampal DA, 5-HT, and norepinephrine (NE) (Sunanda et al., 2000). Additionally, chronic restraint stress (1 h a day for 40 days) affected a reduction of DA and 5-HT levels in the cerebral cortex and the hippocampus of rats (Torres et al., 2002). Our results were in good agreement with their findings; the rats stimulated by 3-h daily restraint stress for 30 days had lower levels of DA and 5-HT in the cerebral cortex and the hippocampus, as compared to the control group. Our results demonstrated that chronic restraint stress affected the decreases of aminergic neurotransmission in the cerebral cortex and the hippocampus. In addition to determining the brain neurotransmitters, our study also investigated blood cortisol levels. Cortisol is well-established as the body's stress hormone. The regulation of cortisol secretion is controlled by the activity of the hypothalamic-pituitary-adrenal (HPA) axis (Jankord and Herman, 2008). Cortisol is an important glucocorticoid hormone secreted by the adrenal cortex. It is well-recognized as the most important hormone intently associated with emotion and cognitive function (Ranabir and Reetu, 2011; Aeran and Hongbum, 2013). The difference in cortisol levels was found in patients with anxiety and depression (Hek et al., 2013; Vreeburg et al., 2009). Hence, the blood cortisol levels in rats were also investigated in our study. Our results demonstrated that 3-h restraint stress for 30 days significantly elevated blood cortisol levels in the stressed rats. Moreover, the treatment with *A. indica* flower extract reduced blood cortisol levels in the stressed rats. Therefore, *A. indica* flower extract could be used to reduce stress in rats.

The results gained from the FST showed that the treatment of *A. indica* flower extract at all doses used in this study reduced immobility time in the stressed rats. These results suggested that *A. indica* flower extract had the property as an antidepressant. Furthermore, the administrations of *A. indica* flower extract also showed significantly enhancement in brain DA and 5-HT levels. The results obtained from the EPMT showed that the rats administered orally with *A. indica* flower extract at a medium dose of 500 mg/kg for 15 and 30 consecutive days had significantly higher percentage of the number of open arm entries and the duration spent in the open arms. These results indicated the anxiolytic effect of *A. indica* flower extract. In addition, *A. indica* flower extract did not produce any psychostimulant effect as observed in the OFT. These results were in good agreement with the study by Jaiswal and collogues who reported that *A. indica* leaf extract exerted anxiolytic activity based on EPMT and OFT (Jaiswal et al., 1994). However, the active compounds in *A. indica* flower and leaf extracts might be different. The compounds found in *A. indica* flower extract employed in this work were shown in Supplementary data. Recently, Duangjai and co-authors reported that quercetin was the main component in *A. indica* flower extract (Duangjai et al., 2019). In our study, the amount of quercetin in *A. indica* flower extract was 7.654 ppm. This finding was in concurrence with the study of Samed and colleagues who reported that quercetin could protect against immobilization stress-induced anxiety, depression, and cognitive function in mice. The administration of quercetin also enhanced 5-HT levels in the brain (Samad et al., 2018). Therefore, quercetin might be the main active ingredient of *A. indica* flower extract to reduce anxiety and depression in the stressed rats in our study. Nevertheless, only the medium dose of *A. indica* flower extract showed an anxiolytic effect. Hence, the medium dose might contain a suitable amount of active compounds to produce an anxiolytic effect. Anxiety and depression involve not only DA and 5-HT, but also GABA (γ-aminobutyric acid) and glutamate (Möhler et al., 2012; Sanacora et al., 2012). Therefore, the evaluation of the effect of other neurotransmitters on anxiety and depression is still needed.

Our work also determined the effect of *A. indica* flower extract on toxic symptoms and mortality rate in rats. The toxic symptoms are the serious symptoms of the rats after administered orally poisoning food or drug (Rice et al., 2018). Behavioral toxicities of harmful substances following oral ingestion in rats were nosebleed, sedation, hypoactivity, loss of appetite (weight loss), dizziness, dyspnea, convulsion, and hyperventilation (Rice et al., 2018). Our results demonstrated that *A. indica* flower extract was a nontoxic substance when given acutely. However, *A. indica* flower extract at a high dose could have a sedative effect and resulted in hypo-activity in rats. Finally, we investigated the gross pathology, morphology, and histology of the liver in rats. Our data showed no abnormality of the liver in all groups. Nevertheless, we did not evaluate histopathology of kidney, blood chemistry for liver, and kidney function tests. Hence, safety information of *A. indica* flower extract should be further studied.

5. Conclusion

The administration of *A. indica* flower extract attenuated stress-induced behavior impairment by the regulation of dopaminergic and serotonergic functions to produce anxiolytic and antidepressant activities. Overall, the present work provides preliminary data on the anxiolytic and antidepressant activities of *A. indica* flower extract in the stressed rats that should be helpful for further studies of this phytomedicine.

Declarations

Author contribution statement

Thaneeya Hawiset: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Napatr Sriraksa: Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Utcharaporn Kamsrijai, Keerati Wanchai: Performed the experiments.

Prachak Inkaew: Analyzed and interpreted the data; Wrote the paper.

Funding statement

This work was supported by Mae Fah Luang University (Grant No: 631B70009).

Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2022.e08881.

Acknowledgements

The authors gratefully acknowledge Assoc. Prof. Dr. Acharaporn Duangjai from the School of Medical Sciences, University of Phayao, Thailand, for providing *A. indica* flower extract. We also would like to thank Dr. Wannisa Sukhorum from the School of Medicine, Mae Fah Luang University, Thailand for histological assessment. Research facilities and financial support (Grant No: 631B70009) from Mae Fah Luang University are greatly appreciated.

References

Aeran, L., Hongbum, C., 2013. The effects caused by lavender and rosemary for salivary cortisol, stress levels and mood alteration. J. Fash. Bus. 17, 18–27.

Alzohairy, M.A., 2016. Therapeutics role of *Azadirachta indica* (Neem) and their active constituents in diseases prevention and treatment. Evid. Based Complement. Alternat. Med. 2016, 7382506.

Baum, A., 1990. Stress, intrusive imagery, and chronic distress. Health Psychol. 9, 653–675.

Chaisawangwong, W., Gritsanapan, W., 2009. Extraction method for high free radical scavenging activity of Siamese neem tree flowers. Songklanakarin J. Sci. Technol. 31, 419–423.

Chu, X., Zhou, Y., Hu, Z., Lou, J., Song, W., Li, J., Liang, X., Chen, C., Wang, S., Yang, B., Chen, L., Zhang, X., Song, J., Dong, Y., Chen, S., He, L., Xie, Q., Chen, X., Li, W., 2016. 24-hour-restraint stress induces long-term depressive-like phenotypes in mice. Sci. Rep. 6, 32935.

Duangjai, A., Nuengchanong, N., Lee, L.H., Goh, B.H., Sakaew, S., Suphrom, N., 2019. Characterisation of an extract and fractions of *Azadirachta indica* flower on cholesterol lowering property and intestinal motility. Nat. Prod. Res. 33, 1491–1494.

Eid, A., Jaradat, N., Elmarzugi, N., 2017. A review of chemical constituents and traditional usage of Neem plant (*Azadirachta indica*). Pal. Med. Pharm. J. 2, 75–81.

Hall, C.S., 1934. Emotional behavior in the rat. I. Defecation and urination as measures of individual differences in emotionality. J. Comp. Psychol. 18, 385–403.

Hek, K., Direk, N., Newson, R.S., Hofman, A., Hoogendijk, W.J., Mulder, C.L., Tiemeier, H., 2013. Anxiety disorders and salivary cortisol levels in older adults: a population-based study. Psychoneuroendocrinology 38, 300–305.

Jaiswal, A.K., Bhattacharya, S.K., Acharya, S.B., 1994. Anxiolytic activity of *Azadirachta indica* leaf extract in rats. Indian J. Exp. Biol. 32, 489–491.

Jankord, R., Herman, J.P., 2008. Limbic regulation of hypothalamo-pituitary-adrenocortical function during acute and chronic stress. Ann. N. Y. Acad. Sci. 1148, 64–73.

Joo, Y., Choi, K.M., Lee, Y.H., Kim, G., Lee, D.H., Roh, G.S., Kang, S.S., Cho, G.J., Choi, W.S., Kim, H.J., 2009. Chronic immobilization stress induces anxiety- and depression-like behaviors and decreases transthyretin in the mouse cortex. Neurosci. Lett. 461, 121–125.

Liu, Y., Zhao, J., Guo, W., 2018. Emotional roles of mono-aminergic neurotransmitters in major depressive disorder and anxiety disorders. Front. Psychol. 9, 2201.

Lopez-Munoz, F., Alamo, C., 2009. Monoaminergic neurotransmission: the history of the discovery of antidepressants from 1950s until today. Curr. Pharmaceut. Des. 15, 1563–1586.

Mah, L., Szabuniewicz, C., Fiocco, A.J., 2016. Can anxiety damage the brain? Curr. Opin. Psychiatr. 29, 56–63.

Martin, E.I., Ressler, K.J., Binder, E., Nemeroff, C.B., 2009. The neurobiology of anxiety disorders: brain imaging, genetics, and psychoneuroendocrinology. Psychiatr. Clin. 32, 549–575.

Masand, P.S., Gupta, S., 2002. Long-term side effects of newer generation antidepressants: SSRIS, venlafaxine, nefazodone, bupropion, and mirtazapine. Ann. Clin. Psychiatr. 14, 175–182.
Matuszewich, L., Filon, M.E., Finnc, D.A., Yamamoto, B.K., 2002. Altered forebrain neurotransmitter responses to immobilization stress following 3,4-methylenedioxymethamphetamine. Neuroscience 110, 41–48.
Möhler, H., 2012. The GABA system in anxiety and depression and its therapeutic potential. Neuropharmacology 62, 42–53.
Pellow, S., Chopin, P., File, S.E., Briley, M., 1985. Validation of open: closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J. Neurosci. Methods 14, 149–167.
Perveen, T., Zehra, S.F., Haider, S., Akhtar, N., Haleem, D.J., 2003. Effects of 2 hrs. restraint stress on brain serotonin metabolism and memory in rats. Pak. J. Pharm. Sci. 16, 27–33.
Porsolt, R.D., Le Pichon, M., Jalfre, M., 1977. Depression: a new animal model sensitive to antidepressant treatments. Nature 266, 730–732.
Ranabir, S., Reetu, K., 2011. Stress and hormones. Indian J. Endocrinol. Metab. 15, 18–22.
Rice, N.C., Rauscher, N.A., Langston, J.L., Myers, T.M., 2018. Behavioral toxicity of sodium cyanide following oral ingestion in rats: dose-dependent onset, severity, survival, and recovery. Food Chem. Toxicol. 114, 145–154.
Rothschild, A.J., 2000. Sexual side effects of antidepressants. J. Clin. Psychiatr. 61, 28–36.
Sanacora, G., Treccani, G., Popoli, M., 2012. Towards a glutamate hypothesis of depression: an emerging frontier of neuropsychopharmacology for mood disorders. Neuropharmacology 62, 63–77.
Samad, N., Saleem, A., Yasmin, F., Shehzad, M.A., 2018. Quercetin protects against stress-induced anxiety- and depression-like behavior and improves memory in male mice. Physiol. Res. 67, 795–808.
Sen, P., Mediratta, P.K., Ray, A., 1992. Effects of *Azadirachta indica* A Juss on some biochemical, immunological and visceral parameters in normal and stressed rats. Indian J. Exp. Biol. 30, 1170–1175.
Sriraksa, N., Kongsui, R., Thongrong, S., Duangjai, A., Hawiset, T., 2019. Effect of *Azadirachta indica* flower extract on functional recovery of sciatic nerve crush injury in rat models of DM. Exp. Ther. Med. 17, 541–550.
Sunanda, Rao, B.S., Raju, T.R., 2000. Restraint stress-induced alterations in the levels of biogenic amines, amino acids, and AChE activity in the hippocampus. Neurochem. Res. 25, 1547–1552.
Suvrathan, A., Tomar, A., Chattarji, S., 2010. Effects of chronic and acute stress on rat behaviour in the forced-swim test. Stress 13, 533–540.
Thippeswamy, B.S., Mishra, B., Veerapur, V.P., Gupta, G., 2011. Anxiolytic activity of *Nymphaea alba Linn.* in mice as experimental models of anxiety. Indian J. Pharmacol. 43, 50–55.
Torres, I.L., Gamaro, G.D., Vasconcellos, A.P., Silveira, R., Dalmaz, C., 2002. Effects of chronic restraint stress on feeding behavior and on monoamine levels in different brain structures in rats. Neurochem. Res. 27, 519–525.
Vreeburg, S.A., Hoogendijk, W.J., van Pelt, J., Derijk, R.H., Verhagen, J.C., van Dyck, R., Smit, J.H., Zitman, F.G., Penninx, B.W.J.H., 2009. Major depressive disorder and hypothalamic-pituitary-adrenal axis activity: results from a large cohort study. Arch. Gen. Psychiatr. 66, 617–626.
Waliullah, S., Javed, K., Jafri, M.A., Singh, S., 2008. Effect of *Azadirachta indica* flower extract on basal and experimentally elevated blood glucose in rats. Orient. Pharm. Exp. Med. 8, 302–310.
Wanchai, K., Hawiset, T., Khumsrijai, U., Sriraksa, N., 2021. *Azadirachta indica* flower extract attenuates kidney oxidative stress in rat exposed to restraint stress. Thai Pharm. Health Sci. J. 16, 380–385.
Whiteford, H.A., Degenhardt, L., Rehmetal, J., 2013. Global burden of disease attributable to mental and substance use disorders: findings from the Global Burden of Disease Study 2010. Lancet 382, 1575–1586.
World Health Organization (WHO), 2001. The World Health Report: 2001: Mental Health: New Understanding, New Hope. World Health Organization, Geneva, Switzerland.